



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> A61K 45/05, 39/00, 37/48 A61K 37/62, C12N 9/02	<b>A1</b>	<b>(11) International Publication Number:</b> WO 93/23080 <b>(43) International Publication Date:</b> 25 November 1993 (25.11.93)
<b>(21) International Application Number:</b> PCT/US93/04582 <b>(22) International Filing Date:</b> 13 May 1993 (13.05.93) <b>(30) Priority data:</b> 07/882,478 13 May 1992 (13.05.92) US <b>(71) Applicant (for all designated States except US):</b> THE BETH ISRAEL HOSPITAL ASSOCIATION [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US). <b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> FOSSEL, Eric, T. [US/US]; 66 Priscilla Road, Chestnut Hill, MA (US). <b>(74) Agent:</b> LORUSSO & LOUD; 440 Commercial Street, Boston, MA 02109 (US).		<b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, US. European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> TARGETED ACTIVATED SPECIES CYTOTOXICITY  <b>(57) Abstract</b>  This invention comprises a method of treating animals, including humans, for conditions such as cancer by producing discrete site cytotoxic environment in an animal, including a human, by the steps of administering to the animal a therapeutically effective dosage of a prooxygenator-affixation element complex; in conjunction with, administering to the animal a therapeutically effective amount of an oxygen source substrate thus producing oxygen free radical species including superoxide at the site of binding. The present invention further comprises a prooxygenator-affixation element complex. In one embodiment the prooxygenator aspect is xanthine oxidase, the affixation element is a tumor specific antibody and the oxygen source substrate is xanthine.		

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1 TARGETED ACTIVATED SPECIES CYTOTOXICITY  
23 Field of the Invention.  
4

5 This invention comprises a method of treating animals,  
6 including humans, for conditions such as cancer by producing a  
7 discrete site cytotoxic environment in an animal, including a  
8 human, by the steps of administering to an animal a  
9 therapeutically effective dosage of a proöxygenator-affixation  
10 element complex; in conjunction with, administering to the animal  
11 a therapeutically effective amount of an oxygen source substrate  
12 thus producing oxygen free radical species including superoxides  
13 at a cytotoxic level at the site of complex binding. The present  
14 invention further comprises a proöxygenator-affixation element  
15 complex. In one embodiment the proöxygenator moiety is xanthine  
16 oxidase, the affixation element is a tumor specific antibody and  
17 the oxygen source substrate is xanthine.

18 Background of the Invention.

19 The earliest medicinal agents were administered either  
20 typically or by ingestion with little control over the site of  
21 drug action. The discovery of penicillin brought the "magic  
22 bullet" to the practice of medicine. Since then pharmacology has  
23 continued to refine techniques to bring the active agents into  
24 the closest proximity with the site of action. For example,  
25

1 today, radio labeled antibodies are used to localize sites in  
2 diagnostic procedures. Similarly, IL-2 binding sites have been  
3 linked to diphtheria toxin to target and destroy activated  
4 T-cells. However, this last approach has been limited to cell by  
5 cell killing of those cells which actually phagocytize the  
6 diphtheria toxin molecule.

7 A major problem with chemotherapy is toxicity.  
8 Chemotherapeutic agents are characterized by high toxicity. This  
9 toxicity is only slightly discriminatory, and, as a general  
10 principal, attacks the entire body injuring or destroying both  
11 normal and abnormal tissue. Many solid tumors are well  
12 vascularized. However cellular antitumor agents have difficulty  
13 reaching tumor cells, in part, due to a fibrin barrier. In  
14 certain instances, the fibrin barrier may be eliminated by  
15 thrombolytic agents. In other instances, treatment of cancers,  
16 and particularly solid tumors, is hampered by inadequate  
17 circulatory investment of such tumors. In fact, the most rapidly  
18 growing tumors may be the most difficult ones in which to obtain  
19 therapeutic concentrations of anti-tumor agents.

20 The present invention is, in its preferred embodiment,  
21 directed to providing the delivery of one or more therapeutic  
22 agents quite specifically to a given site, but not so  
23 specifically that only a given individual cell is treated. The  
24 therapeutic agents are activated oxygen species (collectively,  
25

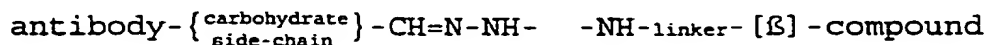
1 "AOS"). AOS of the present invention include peroxides such as  
2 hydrogen peroxide ( $H_2O_2$ ) and superoxide ( $O_2^-$ ), and singlet oxygen  
3 ( $^1O_2$ ). It is just such AOS that have been conjectured as active  
4 agents in polymorphonuclear neutrophils after activation by  
5 pathogens, cytokines or other cell activators.

6 This invention utilizes the known technology of binding  
7 compounds to antibodies so that neither the ability of the  
8 antibody to bind to antigen nor the activity of the bound  
9 compound is impaired. An examples of this technology are U.S.  
10 Pat. No. 4,671,958 issued to Rodwell et al., and U.S. Pat. No.  
11 4,867,973 to Goers et al, the teachings of each being  
12 incorporated herein by reference. U.S. Pat. No. 4,671,958  
13 describes a method for site specific covalent attachment of a  
14 compound to an antibody molecule by selectively oxidizing a  
15 carbohydrate moiety of the antibody, located outside the antigen  
16 binding region of the antibody, to form an aldehyde group with an  
17 amine group (such as a primary amine, secondary amine, hydrazine,  
18 hydrazide, hydroxylamine, phenylhydrazine or semicarbazide) to  
19 form a Schiff base (e.g., oxime, hydrazone, phenylhydrazone, or  
20 semicarbazone, respectively).

21 Accordingly, substrate linkers are modified by attaching  
22 hydrazine or hydrazide derivatives to one end of the linker. The  
23 unmodified sites on the linker may or may not be covalently  
24 attached to a compound. Linkers are synthetic or naturally  
25

1 occurring substrates which are susceptible to cleavage by any of  
2 the components of complement. A number of such linkers are  
3 described and disclosed in U.S. Pat. No. 4,671,958, including  
4 N-Boc-tyrosine o-nitrophenyl ester, N-acetyl-gly-lys-methyl ester  
5 and others well known in the art.

6 By way of example, substrate linkers which are attached to a  
7 compound via an ester or amide link, are modified by attaching a  
8 hydrazide such as phenylhydrazine to the opposite amino terminus  
9 of the peptide chain. The hydrazide derivative of the peptide  
10 linker is attached to a compound via an ester or amide link is  
11 then reacted with an oxidized immunoglobulin fragment containing  
12 an oxidized carbohydrate. This results in hydrazone formation  
13 and the covalent attachment of the compound to the carbohydrate  
14 side chain of the immunoglobulin via a linker group which is  
15 susceptible to cleavage by complement. The described covalent  
16 attachment of linker to the carrier antibody does not interfere  
17 with the antigen binding site of the molecule nor with complement  
18 fixation. Schematically this may be represented:



20 where ß represents an amide or ester bond.

21 Summary of the Invention.

22 This invention includes a proöxygenator-affixation element  
23 complex. In particular embodiments the proöxygenator-affixation  
24 element complex comprises a proöxygenator moiety of at least one  
25

1 enzyme, such as xanthine oxidase, superoxide dismutase, or a  
2 myeloperoxidase. In specific embodiments of the invention the  
3 proöxygenator-affixation element complex comprises an affixation  
4 element being an antibody. Particular antibodies are those that  
5 bind to melanoma, carcinoma, adenocarcinoma, sarcoma,  
6 neuroblastoma, myeloma, lymphoma, or leukemia cells. Examples  
7 within the invention are antibodies such as  $\alpha$ -MSH,  
8 carcino-embryonic antigen,  $\alpha$ -fetoprotein, or SSEA-1. Other  
9 examples are wherein proöxygenator-affixation element complex  
10 comprises an affixation element being a peptide such as the  
11 diphtheria fragment B, or IL-2 binding site.

12  
13 This invention also includes a method of producing discrete  
14 site cytotoxic environment in an animal, including a human,  
15 comprising the steps of:

16 (i) administering to said animal a therapeutically effective  
17 dosage of a proöxygenator-affixation element complex wherein said  
18 complex has a binding affinity for the site of cytotoxic  
19 environment production; and thereafter,

20 (ii) administering to said animal a therapeutically  
21 effective amount of an oxygen source substrate. In a particular  
22 embodiment, upon administration, the affixation element of the  
23 proöxygenator-affixation element complex performs the step of  
24 binding the complex to a cell. Some proöxygenator elements of  
25 the method are xanthine oxidase, superoxide dismutase, or

1 myeloperoxidase. Certain oxygen source substrates of the method  
2 are methylxanthines such as xanthine, caffeine or theophylline.  
3 The method of this invention also encompasses the step of  
4 maintaining the AOS concentration in a discrete area to at least  
5 about  $10^{-8}$  M/minute for a particular intervals of at least about  
6 15 minutes, and preferably at least about  $10^{-6}$  M/minute for a  
7 particular interval or intervals of at least about 15 minutes,  
8 and more preferably at least about  $10^{-5}$  M/minute for a  
9 particular intervals of at least about 15 minutes. In particular  
10 aspects the method includes administering to an animal a  
11 therapeutically effective dosage of a proöxygenator-affixation  
12 element complex which comprises binding said complex to at least  
13 about 50% and preferably at least about 80% of the binding sites  
14 at the general site of cytotoxic environment production.

15 In a diagnostic application, this invention includes adding  
16 to a tissue culture of a tumor to be tested two or more graduated  
17 dosages of a proöxygenator-affixation element complex wherein  
18 said complex has a binding affinity for the tumor being tested;  
19 and thereafter,

20 administering to said culture a therapeutically effective  
21 amount of an oxygen source substrate;

22 determining tumor growth inhibition in said tissue culture.

23 Detailed Description of the Invention.  
24  
25



1           This invention will best be understood with reference to the  
2 following definitions:

3           A. Proöxygenator shall mean at least one moiety which  
4 produces an AOS upon exposure to at least one oxygen bearing  
5 substrate.

6           In some applications it will be appreciated that multiple  
7 proöxygenator moieties may be attached to a single affixation  
8 moiety. These may be the same proöxygenator moieties or  
9 different proöxygenator moieties. In one example, xanthine  
10 oxidase and a peroxidase such as superoxide dismutase may be  
11 cojoined to a single affixation moiety. Additionally, marker  
12 moieties such as radio labels, fluorescent materials or NMR  
13 labels may be affixed.

14           B. AOS of the present invention shall mean activated oxygen  
15 species including peroxides such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and  
16 oxygen free radicals, ( $\text{O}_2^\cdot$ ),  $\text{HO}^\cdot$ , and  $\text{HOO}^\cdot$ . The particular AOS,  
17  $\text{O}_2^\cdot$ , is termed "superoxide."

18           AOS of this invention further include singlet oxygen ( $^1\text{O}_2$ ).

19           Paradigm reactions of this invention are (1) the conversion  
20 of xanthine to superoxide, the oxygen free radical ( $\text{O}_2^\cdot$ ) by the  
21 enzyme xanthine oxidase, and (2) the conversion of xanthine to  
22 uric acid and superoxide, an oxygen free radical ( $\text{O}_2^\cdot$ ).

1 Without being bound to a particular theory, it is believed  
2 that the efficacy of this invention is a consequence of the  
3 provision of AOS in therapeutically effective concentrations to  
4 the site of complex binding. While not bound by any particular  
5 scheme by which the provision of AOS to the site of complex  
6 binding provide therapeutic efficacy, it is believed that the  
7 desired reaction such as tumor toxicity is substantially similar  
8 to the cytotoxic and bactericidal system found in  
9 polymorphonuclear neutrophil leukocyte (PMN). In PMN systems,  
10 researchers have found that ( $O_2^-$ ),  $HO\cdot$ , and  $HOO\cdot$  are directly  
11 cytotoxic. In addition  $H_2O_2$  may react with  $Cl^-$  to form  $OCl^-$   
12 (hypochlorite ion) which is a bactericidal agent. In addition to  
13 hydrogen peroxide, the oxygen radical, singlet oxygen ( $O_2^1$ ), and  
14 hydroxy radical ( $HO\cdot$ ) are also associated with  
15 bactericidal/anti-pathogen activity. In a similar fashion,  
16 macrophages taken from BCG-infected animals or otherwise  
17 activated have been reported as destroying tumor cells in tissue  
18 culture through elaboration of hydrogen peroxide and tumor  
19 necrosis factor.

20 C. Affixation element shall mean a cell receptor site  
21 moiety such as an antibody or peptide capable of affixing the  
22 complex to a site on a cell. The affixation element of the  
23 complex is understood to have a binding affinity for the site of  
24 cytotoxic environment production. This can be at a site  
25

1 particular to a tumor, but also particular to certain classes of  
2 cells such as interleukin binding sites. An affixation element  
3 will also be required to cojoin at least one proöxygenator moiety  
4 and preferably more than one such moiety. Examples of affixation  
5 elements are the cell binding fragment of diphtheria toxin  
6 (fragment B), the IL-2 binding site, and antitumor antibodies  
7 such as  $\alpha$ -MSH.

8 It is understood that in the practice of this invention,  
9 some sites undergo phagocytosis. That is the site of cellular  
10 affixation which is initially external becomes drawn into the  
11 cell. While it is preferred that the cell bound  
12 proöxygenator-affixation element complex remain external to the  
13 cell, this is not an absolute requirement. Antibodies are a  
14 particular category of affixation element, generally comprising  
15 proteins circulating in plasma.

16 D. Complex shall mean a proöxygenator moiety bound to an  
17 affixation element such that (1) the proöxygenator moiety remains  
18 capable of enzymatically converting an oxygen source substrate  
19 into AOS, and (2) the affixation element as complexed to the  
20 proöxygenator moiety maintains specificity for the target site of  
21 affixation.

22 E. Xanthine oxidase shall mean the enzyme  
23 xanthine:oxygen oxidoreductase, an iron-molybdenum flavoprotein.  
24  
25

1 F. Discrete site cytotoxic environment shall mean the  
2 provision of a cytotoxic environment at a defined location  
3 proximate to a proöxygenator-affixation element complex bound to  
4 a cell, but not limited to the single bound cell.

5 G. Cytotoxic environment shall mean an environment that  
6 results in reduction or cessation of proliferation of a cell type  
7 and further may include death of some or all cells of a given  
8 cell type. Cell is used as an inclusive term encompassing  
9 differentiated tissue, single cells, bacteria, multicellular  
10 pathogenic organisms, viri, retroviri, and neoplastic cells.

11 Cytotoxic environment shall further be expansively  
12 understood to include AOS as a "neo-adjuvant," that is as a  
13 potentiator of other therapies. The neo-adjuvant function is  
14 displayed in conjunction with other therapy such as radiation,  
15 chemotherapy, and vaccine/immunomodulation therapy -- each of  
16 which is potentiated by cellular changes including permeability  
17 changes and protein expression/recognition changes resulting from  
18 the practice of this invention.

19 H. Tumor specific antibody shall mean an antibody that  
20 preferentially binds to neoplastic cells. In particular  
21 embodiments, antibodies to malignant melanoma, carcinoma,  
22 adenocarcinoma, sarcoma (including, Kaposi sarcoma),  
23 neuroblastoma, myeloma, lymphoma, and leukæmias.

1 I. Xanthine shall refer to methylxanthines and analogues  
2 and derivatives thereof. This shall be understood to include,  
3 without limitation, hypoxanthine, caffeine, theophylline,  
4 theobromine, dysphylline, enprofylline, and pentoxifylline.

5 J. Therapeutically effective shall mean a dosage that  
6 produces the desired physiological effect. As to a  
7 proöxygenator-affixation element complex, therapeutically  
8 effective means that sufficient complex is bound such that when  
9 presented with oxygen bearing substrate a cytotoxic environment  
10 arises. In the practice of the method of this invention two  
11 steps are required. First the complex must be bound to the  
12 target cells in therapeutically effective concentration -- which  
13 is necessarily a potential for physiological activity realized as  
14 permanent effect only upon the presentation of oxygen bearing  
15 substrate. Therapeutically effective as to a dosage of oxygen  
16 bearing substrate shall be one sufficient to establish a  
17 cytotoxic environment at the site of complex binding in the  
18 presence of bound complex. Such dosage provides an environment  
19 that results in reduction or cessation of proliferation of a cell  
20 type and further may include death of some or all cells of a  
21 given cell type or at a given location.

22 In the practice of this invention it will be of importance  
23 to select an affixation element that will bind to a target cell  
24 in sufficient concentration to ultimately provide therapeutically  
25

1 effective AOS concentration at the target site. While some  
2 pathogens are exquisitely sensitive to AOS others are  
3 recalcitrant. Binding of complex at a high density of sites at a  
4 high saturation for a lengthy period will be factors tending to  
5 increase obtainable AOS levels. Other factors are the number of  
6 proöxygenator moieties bound to each antibody, the activity of  
7 each proöxygenator moiety, and availability of AOS substrate and  
8 the absence of competitive or inhibitory reactants.

9 Tumor Specific Antigens: Tumor cells can frequently be  
10 targeted by antigenic determinants. Cells infected with  
11 oncogenic viri frequently have two recognition antigens displayed  
12 on the cell surface, either of which may provide suitable sites  
13 for antibody binding. Oncofetal antigens may be expressed on the  
14 surface tumor cells which differentiate adult tissues from tumor  
15 tissues. Examples of these are carcino-embryonic antigen (CEA)  
16 in cancer of the intestine and  $\alpha$ -fetoprotein in hepatic  
17 carcinoma. There are available monoclonal antibodies raised  
18 against human melanoma cells that also react with tumors of  
19 neural origin. Another monoclonal antibody defines the SSEA-1  
20 antigen found on a variety of human tumors. Tumors induced by  
21 chemical agents such as benzopyrene have tumor specific antigens.  
22 Researchers have particularly noted the tumor specificity of the  
23  
24  
25

1 Ig idiotype on the surface of chronic leukæmic cells. Other  
2 tumor specific antigens can be prepared by methods well known in  
3 the art and do not comprise a part of this invention.

4 Tumors sensitive to the AOS therapy of this invention, and  
5 therapeutically effective dosage levels may be determined by *in*  
6 *vitro* techniques which are known in the art. For example, a  
7 tumor may be conveniently grown in tissue cultures. To the  
8 tissue cultures a variety of proöxygenator-affixation element  
9 complexes at a variety of concentrations may be presented with  
10 various oxygen source substrates in a checker board assay or the  
11 like. The most inhibited tissue cultures will define the  
12 therapeutically effective complexes, oxygen source substrates,  
13 and may be extrapolated to define a range of therapeutically  
14 effective dosages. Additional agents may be cross tested in, for  
15 example, traditional *in vitro* Combination Effect Test or the  
16 Therapeutic Index Test, to determine if neo-adjuvant activity may  
17 be advantageously used as well.

18 The Combination Effect Test employs a series of tests to  
19 determined combined drug efficacy. One such test is the "Checker  
20 Board Assay" to test different serial dilutions of the drugs to  
21 be combined with AOS administration as challenged by a test cell  
22 culture of cancer cells in agar or broth. Another test is the  
23 Virus Titer Reduction Assay, measuring the reduction in  
24 multiplication of virus as grown in host cells. Another test is  
25

1 an increase in the therapeutic index which is the dose lethal to  
2 50% of the subjects as compared to the dose therapeutically  
3 effective in 50% of the cases. The use of the Combination Effect  
4 Test allows for the coadministration of AOS with other drugs in a  
5 useful and efficacious manner. Particular reference is made to  
6 the increased efficacy of Tumor Necrosis Factor by the practice  
7 of this invention.

8  
9       Complexing a Proöxygenator with an Affixation Element: In  
10 combining a proöxygenator moiety with an affixation element care  
11 must be taken to preserve the AOS forming activity (usually  
12 enzymatic) of the proöxygenator and the binding strength and  
13 specificity of the affixation element. To accomplish complexing  
14 either chemical or recombinant methods may be usefully employed.  
15 As a cojoining methodology, hybridizing IL-2 with a toxin has  
16 been described in Greenfield et al., "Science," pp 238, 536  
17 (1979). Also, hybridization of diphtheria toxin/IL-2 has been  
18 described in U.S. Pat. No. 4,675,382 using recombinant DNA  
19 methodologies. Pseudomonas exotoxin A/IL-2 hybridization has  
20 been described in Lorberboum-Galski et al., "Proc. Natl. Acad.  
21 Sci. USA 85: 1922-26, (1988). The teachings of the foregoing  
22 references are incorporated herein by reference. In, for  
23 example, Lorberboum-Galski et al., IL-2 replaced the endogenous  
24 cell-specific receptor domain of the toxin protein, Pseudomonas  
25 exotoxin A/IL-2. Further examples of this technology are set



1 forth in US Patent 5,047,227 to Rodwell, "Novel and Improved  
2 Antibodies for Site Specific Attachment of Compounds;" US  
3 Patent 4,937,183 to Ultee et al., "Method for Preparation of  
4 Antibody-fragment Conjugates;" US Patent 4,867,973 to Goers et  
5 al., "Antibody-Therapeutic Agent Conjugates;" and US Patent  
6 4,671,958 to Rodwell et al., "Antibody Conjugates for the  
7 Delivery of Compounds to Target Sites" the teachings of which are  
8 incorporated herein by reference. Similar information is  
9 set forth in European Patent Application 90311590.5, Publication  
10 No. 425,235 A2, by Chari et al. the teachings of which are  
11 incorporated herein by reference.

12       The compositions and methods of this invention possess  
13 valuable pharmacological properties. The proöxygenator-affixation  
14 element complex can localize on or near such targets as tumors  
15 cells, cysts, areas of inflammation, and individual viri or  
16 retroviri. In the presence of an oxygen source substrate, the  
17 proöxygenator-affixation element complex will provide discrete  
18 site cytotoxic environment. Such discrete site cytotoxic  
19 environment will retard or reverse growth of the target cells or  
20 organisms. In some applications the desired effect will further  
21 include cytotoxic treatment of other nearby cells or organisms at  
22 the same discrete site. The discrete site cytotoxic effect is of  
23 great benefit in the field of medicine, particularly in the field  
24 of cancer therapy. This benefit is demonstrated, for example,  
25

1 using the method of administering a complex of tumor specific  
2 antibody-xanthine oxidase in conjunction with administration of  
3 xanthine. A cytotoxic environment at the tumor site is  
4 established to preferentially kill tumor cells, with minimal off  
5 site toxicity.

6 Thus, these compositions can be used with indications  
7 providing a binding site for the complex. Included indications  
8 are solid tumor neoplasms as well as systemic neoplasms including  
9 cancers, leukemias, viral diseases wherein the virus is  
10 "recognized" and attached by the antibody, brucellosis,  
11 shistomiasis, malaria, and bacterial infections.

12 The compositions and method are particularly useful as  
13 antitumor agents wherein the tumor is strongly antigenically  
14 identifiable by the antibody of the complex and wherein the tumor  
15 is susceptible to AOS. The composition can be used in  
16 conjunction with other therapeutic agents as a neo-adjuvant.

17 In addition, the compositions can be used in *in vitro*  
18 diagnostics for determining which target cells are sensitive or  
19 susceptible to treatment via AOS (alone or in combination with  
20 other drugs) at concentrations obtainable *in vivo*.

21 The compositions of this invention are generally  
22 administered to animals, including but not limited to mammals,  
23 and avians, and particularly, livestock, household pets, humans,  
24 cattle, cats, dogs, poultry, etc.

25

1       The pharmacologically active compositions of this invention  
2       can be processed in accordance with conventional methods of  
3       Galenic pharmacy to produce medicinal agents for administration  
4       to patients, e.g., mammals including humans.

5       The compositions of this invention can be employed in  
6       admixture with conventional excipients, i.e., pharmaceutically  
7       acceptable organic or inorganic carrier substances suitable for  
8       parenteral, enteral (e.g., oral or inhalation) or topical  
9       application which do not deleteriously react with the active  
10      compositions. Suitable pharmaceutically acceptable carriers  
11      include but are not limited to water, and salt solutions (e.g.,  
12      isotonic saline, buffered saline) and injectable formulations  
13      (including i.v., and peritoneal) .

14  
15      The pharmaceutical preparations can be sterilized but must  
16      not be denatured. If desired, pharmaceutical preparations may be  
17      mixed with auxiliary agents, e.g., lubricants, preservatives,  
18      stabilizers, wetting agents, emulsifiers, salts for influencing  
19      osmotic pressure, buffers, and the like which do not  
20      deleteriously react with the active compositions. They can also  
21      be combined where desired with other active agents, e.g.,  
22      prooxygenator-affixation element complex administered with an  
23      oxygen source substrate.

1           For parenteral application, particularly suitable are  
2   injectable, sterile solutions, preferably aqueous solutions, as  
3   well as suspensions, or emulsions. Ampoules are convenient unit  
4   dosages. In certain localized administrations the  
5   proöxygenator-affixation element complex and/or oxygen source  
6   substrate may be administered via intravenous shunt permitting  
7   "up stream" introduction of therapeutic agents and "down stream"  
8   removal of therapeutic agents. Thus, high localized  
9   concentrations of therapeutic agents may be obtained, and yet  
10   maintain low systemic levels.

11           Sustained or directed release compositions can be  
12   formulated, e.g., liposomes, or those wherein the active  
13   component is protected with differentially degradable coatings,  
14   e.g., by microencapsulation, multiple coatings, etc. It is also  
15   possible in certain applications to freeze-dry the new  
16   compositions and use the lyophilates obtained, for example, for  
17   the preparation of products for injection.

18           For topical application such as to the lungs, suitable are  
19   sprayable aerosol preparations wherein the active ingredient,  
20   preferably in combination with a liquid inert carrier material,  
21   is packaged in a squeeze bottle or provided by nebulizer.

1           Intravenous administration is preferred. However, the  
2 specific mode of administration will vary with the site of  
3 treatment and the particular active agents. The method of  
4 administration will preferably be selected to develop the highest  
5 AOS concentration at the site of treatment.

6           Dosages of both the proöxygenator-affixation element complex  
7 administered and the oxygen source substrate(s) may be determined  
8 empirically by methods known to those skilled in the art.  
9 However the method and agents of the instant invention are  
10 uniquely determinable by calculation. An antibody's affinity for  
11 target binding sites is determinable by standard methods.  
12 Similarly, the general number of binding sites in a given  
13 antibody-receptor application of the invention is determinable.

14           In the case of superoxide ( $O_2^-$ ) as produced by xanthine  
15 oxidase, the following calculations are instructive.

- 16 1. Each xanthine throws off one superoxide,  $O_2^-$ .
- 17 2. The specific activity of xanthine oxidase is  $\sim 14,000$ , thus  
18 the enzyme can produce  $14,000 \mu M$  of  $O_2^-$  per minute.
- 19 3. A given cell has about 40,000 binding sites for a given  
20 antibody.
- 21 4. Based on a single cell (and presuming only one enzyme per  
22 antibody), the area local to that cell may have  $5.6 \times 10^8$   
23  $\mu M O_2^-$ /min, or roughly 560M/sec.
- 24 5. The lifetime of superoxide is about  $10^{-6}$  to  $10^{-9}$ .

6. Thus maintained site concentration at an instantaneous sampling is between about  $10^{-5}$  to about  $10^{-8}$  M of superoxide/minute.

Concentration levels can be altered by binding more than one enzyme to an antibody, or utilizing enzymes of increased activity. Further, attachment of antibody and associated enzymatic activity as generally distributed in an area will result in nodes of increased AOS concentration.

While these will vary widely with each antibody, binding site, volume over which antibody complex is distributed and the half-life of the complex, such determinations are within the recognized skill of practitioners in the art. Dosages based on these factors -- bearing in mind tolerable toxicity levels -- will then be determined.

In a like fashion, the dosage and time of administration of oxygen source substrate(s) to form AOS from a complex containing xanthine oxidase may be either determined empirically or calculated. In the example of the methylxanthine, caffeine, as an oxygen source substrate(s), the dosage of caffeine will not exceed the capacity of the xanthine oxidase to form AOS. Calculation will include volume throughout which the xanthine is distributed and the half-life of caffeine in vivo. In the example of caffeine and theophylline, in humans it is known to be distributed into all body compartments, and its apparent

1 distribution is about 0.4 to about 0.6 liter/kg of body weight,  
2 and higher in premature infants. The half-life of caffeine in  
3 plasma is about 3 to 7 hours. Variance in the half-life,  
4 however, in specific circumstances is well known to those skilled  
5 in the art. For example, the half-life may double in women in  
6 the later stages of pregnancy, or be up to 50 hours in premature  
7 infants. There is also well document substantial  
8 inter-individual variation in clearance of methylxanthines, and  
9 such clearance should be tested to determine the individual  
10 dosage requirements. Caffeine dosages typically should not  
11 exceed 15 mg/kg and plasma concentrations of 30 $\mu$ g/ml. Tolerated  
12 methylxanthine dosage levels are well known in the art, such as  
13 are found in Goodman and Gilman's The Pharmacological Basis of  
14 Therapeutics Eighth Edition, Eds., Gilman, Rall, Nies, Taylor  
15 (Pergamon Press, New York, New York, 1990), the teachings of  
16 which are incorporated herein by reference.

17 The dosage of the compositions according to this invention  
18 generally are designed to afford maximal tolerated delivery of  
19 AOS to the target site. It will be appreciated that the actual  
20 preferred amounts of active compositions in a specific case will  
21 vary according to the specific compositions being utilized, the  
22 particular compositions formulated, the mode of application, and  
23 the particular situs and organism being treated. Dosages for a  
24 given host can be determined using conventional considerations,  
25

1 e.g., by customary comparison of the differential activities of  
2 the subject compositions and of a known agent, e.g., by means of  
3 an appropriate, conventional pharmacological protocol.

4  
5 In the practice of this invention utilizing xanthine oxidase  
6 bound to antibody the proöxygenator-affixation element complex  
7 the following steps are taken. A subject in need of AOS  
8 therapeutic treatment and having an antibody specific treatment  
9 site is administered xanthine to a concentration of about  $10^{-9}$  to  
10 about  $10^{-5}$  M. Particular effective concentrations are from about  
11 concentration of about  $10^{-8}$  to about  $10^{-6}$  M, as well as from about  
12 concentration of about  $10^{-6}$  to about  $10^{-5}$  M. If toxicity is at  
13 issue, maximum concentration is established over time, with  
14 xanthine administration curtailed when unsuitable toxicity begins  
15 to be manifested. Maximum concentration is reached about 1 hour  
16 after oral administration. In a 70kg subject, administration of  
17 xanthine in doses of from about 300 mg to 500mg is useful.  
18 Thereafter the proöxygenator-affixation element complex, xanthine  
19 oxidase bound to an antibody specific to the treatment site,  
20 administered intravenously to establish a concentration which  
21 will bind to binding sites in from about 20% to 100% of such  
22 sites. Xanthine oxidase bound to said antibody is periodically  
23 readministered in proportion to the rate at which enzyme-antibody  
24 is deactivated, here about every three hours. Due to the long  
25 half-life of xanthine, it is not usually necessary to



1 readminister xanthine during the course of this treatment. In  
2 particular embodiments it is useful to administer the  
3 proöxygenator-affixation element complex prior to administration  
4 of the substrate.

5 Example 1

6 Xanthine Oxidase/ $\alpha$ -MSH Complex

7 To a human suffering from malignant melanoma, xanthine is  
8 administered intravenously to obtain a plasma level of 10-30 $\mu$ g/ml  
9 which is maintained over 4 hours by additional xanthine  
10 administration as required. Twenty minutes after initial  
11 xanthine administration, a proöxygenator-affixation element  
12 complex consisting of a proöxygenator moiety of xanthine oxidase  
13 and an affixation element of  $\alpha$ -melanocyte stimulating hormone  
14 ( $\alpha$ -MSH) is administered, i.v. The xanthine oxidase/( $\alpha$ -MSH  
15 complex is suspended in isotonic saline. Administration is  
16 intravenous at a dosage of 100 mg every ten minutes until 80% of  
17 the binding sites on target cells are occupied. As used herein  
18 binding cites on target cells refers to the binding of the  
19 proöxygenator-affixation element complex the at the site of  
20 cytotoxic environment production. Such binding results from the  
21 affinity between complex and binding cite. This treatment is  
22 repeated daily for 5 days.

I claim:

1. A proöxygenator-affixation element complex.
2. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises a proöxygenator moiety of at least one enzyme.
3. The complex of Claim 2 wherein the enzyme is a xanthine oxidase.
4. The complex of Claim 2 wherein the enzyme is a superoxide dismutase.
5. The complex of Claim 2 wherein the enzyme is a myeloperoxidase.
6. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises an affixation element being an antibody.
7. The complex of claim 6 wherein the antibody binds to melanoma, carcinoma, adenocarcinoma, sarcoma, neuroblastoma, myeloma, lymphoma, or leukemia cells.

8. The complex of Claim 6 wherein antibody is  $\alpha$ -MSH, carcino-embryonic antigen,  $\alpha$ -fetoprotein, or SSEA-1.
9. The complex of Claim 1 wherein proöxygenator-affixation element complex comprises an affixation element being an peptide.
10. The complex of claim 9 wherein the peptide is the diphtheria fragment B, or IL-2 binding site.
11. A method of producing discrete site cytotoxic environment in an animal, including a human, comprising the steps of  
administering to said animal a therapeutically effective dosage of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the site of cytotoxic environment production; and thereafter,  
administering to said animal a therapeutically effective amount of an oxygen source substrate;  
forming an activated oxygen species (collectively, "AOS").
12. The method of Claim 11 wherein upon administration said affixation element of the proöxygenator-affixation element complex performs the step of  
binding the complex to a cell.

13. The method of Claim 11 wherein the proöxygenator element comprises xanthine oxidase, superoxide dismutase, or myeloperoxidase.

14. The method of Claim 11 wherein the oxygen source substrate is a methylxanthine.

15. The method of Claim 14 wherein the methylxanthine is xanthine.

16. The method of Claim 14 wherein the methylxanthine is caffeine.

17. The method of Claim 14 wherein the methylxanthine is theophylline.

18. The method of Claim 11 further comprising the step of maintaining the AOS concentration in a discrete area to at least about  $10^{-8}$  M/minute for a particular intervals of at least about 15 minutes.

19. The method of Claim 18 further comprising the step of maintaining the AOS concentration in a discrete area to at least about  $10^{-6}$  M/minute for a particular intervals of at least about 15 minutes.

20. The method of Claim 19 further comprising the step of maintaining the AOS concentration in a discrete area to at least about  $10^{-5}$  M/minute for a particular intervals of at least about 15 minutes.

21. A method of Claim 11 wherein

administering to said animal a therapeutically effective dosage of a proöxygenator-affixation element complex comprises binding said complex to at least about 50% of the binding cites at said site of cytotoxic environment production.

22. The method of Claim 21 wherein said binding is to at least about 80%.

23. A method of diagnosing AOS treatable tumors comprising:

adding to a tissue culture of a tumor to be tested two or more graduated dosages of a proöxygenator-affixation element complex wherein said complex has a binding affinity for the tumor being tested; and thereafter,

administering to said culture a therapeutically effective  
amount of an oxygen source substrate;  
determining tumor growth inhibition in said tissue culture.

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/04582

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) : A61K 45/05, 39/00, 37/48, 37/62; C12N 9/02  
US CL : 424/94.2, 94.1, 94.3, 85.1, 85.2, 85.91; 435/189

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.2, 94.1, 94.3, 85.1, 85.2, 85.91; 435/189; 514/410, 185

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CA, Medline, Biosis, Registry

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,906,469 (Jansen, et al.) 06 March 1990, see entire document, especially col. 1, lines 26-31, col. 5, lines 11-12, col. 6, lines 6-8 and 54-64, and col. 13, lines 6-9.	1-23
Y	MOLECULAR AND CELLULAR BIOCHEMISTRY, Vol. 10(1), issued 31 January 1976, A. Bozzi, et al., "Enzyme Defense Against Reactive Oxygen Derivatives. II. Erythrocytes and Tumor Cells," pages 11-16, especially pages 11 and 12.	1-23
Y	US, A, 4,971,991 (Umemura, et al.) 20 November 1990, see entire document.	1-23
Y	US, A, 4,975,278 (Senter, et al.) 04 December 1990, see entire document.	1-23



Further documents are listed in the continuation of Box C.



See patent family annex.

•	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search

21 JULY 1993

Date of mailing of the international search report

29 JUL 1993

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

KRISTIN K. LARSON

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/04582

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,762,707 (Jansen, et al.) 09 August 1988, see entire document.	1-23
A	US, A, 4,937,183 (Ultee, et al.) 26 June 1990.	1-23
A	US, A, 4,671,958 (Rodwell, et al.) 09 June 1987.	1-23
A	US, A, 4,867,973 (Goers, et al.) 19 September 1989.	1-23
A	ACCOUNTS OF CHEMICAL RESEARCH, Vol. 5(10), issued October 1972, I. Fridovich, "Superoxide Radical and Superoxide Dismutase," pages 321-326.	1-23